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Characterization of *bla*_{CMY} Plasmids and Their Possible Role in Source Attribution of *Salmonella enterica* Serotype Typhimurium Infections

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Abstract

Salmonella is an important cause of foodborne illness; however, identifying the source of these infections can be difficult. This is especially true for *Salmonella* serotype Typhimurium which is found in diverse agricultural niches. Extended spectrum cephalosporins (ESC) are one of the primary treatment choices for complicated *Salmonella* infections. In *Salmonella*, ESC resistance in the U.S. is mainly mediated by *bla*_{CMY} genes carried on various plasmids. In this study, we examined whether the characterization of *bla*_{CMY} plasmids, along with additional information, can help us identify potential sources of infection by *Salmonella*, and use serotype Typhimurium as a model. In the U.S., monitoring of retail meat, food animals, and ill persons for antimicrobial resistant *Salmonella* is conducted by the National Antimicrobial Resistance Monitoring System (NARMS). In 2008, 70 isolates (70/581; 12.0 %) (34 isolates from retail meat, 23 food animal, and 13 human) were resistant to ceftriaxone and amoxicillin/clavulanic acid. All were PCR-positive for *bla*_{CMY} and 59/70 (84.3%) of these genes were plasmid-encoded. PCR-based replicon typing (PBRT) identified 42/59 (71.2%) IncI1-*bla*_{CMY} plasmids and 17/59 (28.8%) IncA/C-*bla*_{CMY} plasmids. Isolates from chickens or chicken products with *bla*_{CMY} plasmids primarily had IncI1-*bla*_{CMY} plasmids (37/40; 92.5%), while all isolates from cattle had IncA/C-*bla*_{CMY} plasmids. Isolates from humans had either IncA/C- *bla*_{CMY} (n = 8/12; [66.7%]) or IncI1- *bla*_{CMY} (n = 4/12 [33.3%]) plasmids. All of the IncI1-*bla*_{CMY} plasmids were ST12 or were closely related to ST12. Antimicrobial susceptibility patterns (AST) and pulsed-field gel electrophoresis (PFGE) patterns of the isolates were also compared and differences were identified between isolate sources. When the source of a Typhimurium outbreak or sporadic illness is unknown, characterizing outbreak isolate's *bla*_{CMY} plasmids, AST, and PFGE patterns may help identify it.

Introduction

Salmonella is an important cause of foodborne illness in the United States resulting in approximately 1.2 million cases of salmonellosis a year (Scallan E 2011). Although

salmonellosis is usually self-limiting, severe infections typically require antimicrobial treatment (RedBook 2012). Infections are commonly associated with consuming contaminated food or water. However, identifying the source of these infections can be difficult. This is especially true for *Salmonella enterica* serotype Typhimurium, which is found in diverse agricultural niches and is typically among the top serotypes associated with salmonellosis each year (Centers for Disease and Prevention 2010).

The National Antimicrobial Resistance Monitoring System (NARMS) determines antimicrobial susceptibility of *Salmonella* from humans, retail meats, and food animals. In 2008, *Salmonella* ser. Typhimurium was the most common serotype isolated from retail chicken breasts, the fourth most common from chickens, the fifth most common from cattle, and the second most common serotype from humans (United States Department of Agriculture 2009, United States Food and Drug Administration (A) 2009, Centers for Disease Control and Prevention 2010). Antimicrobial resistance in serotype Typhimurium is associated with bloodstream infection which is of concern because these patients are more likely to require antimicrobial treatment (Crump, Medalla et al. 2011). Extended spectrum cephalosporins (ESC), such as ceftriaxone, are one of the primary treatment choices for invasive salmonellosis (RedBook 2012). However, ESC resistance among *Salmonella* is on the rise in the U.S. and threatens to complicate treatment options (United States Food and Drug Administration (B) 2009). Among ceftriaxone resistant *Salmonella* collected in the United States in 2008, Typhimurium was the second most common serotype found in humans, the first from chicken retail meat, the second from chickens, and the fourth from cattle.

Considerable research has been performed on identifying the mechanisms of cephalosporin resistance in *Salmonella*. In the U.S., cephalosporin resistance is primarily mediated by AmpC β -lactamases, encoded by *bla*_{CMY} genes (Philippon, Arlet et al. 2002, Folster, Pecic et al. 2010, Folster, Pecic et al. 2011). These genes are commonly carried on various types of plasmids, which can be distinguished by their incompatibility/replicon type (Carattoli, Bertini et al. 2005). Previous studies identified two major types of *bla*_{CMY} plasmids among *Salmonella* in the U.S.; IncA/C and IncII (Folster, Pecic et al. 2010, Folster, Pecic et al. 2011). In this study, we examined *bla*_{CMY}-positive Typhimurium isolates from retail meat, food animals, and humans to determine whether the phenotypic and genotypic characteristics of *bla*_{CMY} plasmids can help us identify possible sources of infection by Typhimurium. We chose to focus on serotype Typhimurium isolates due to their commonality and widespread sources, however it is our hope that this study will serve as a model of all *bla*_{CMY}-positive *Salmonella* serotypes.

Methods

Isolate collection and testing

Salmonella isolates from ill persons were obtained from specimens submitted to clinical laboratories in the United States and forwarded to state public health laboratories. Participating state public health laboratories serotyped and submitted every twentieth non-typhoidal *Salmonella* (NTS) to the CDC NARMS laboratory for susceptibility testing. NARMS retail meat monitoring was conducted by the United States FDA-Center for

Veterinary Medicine as previously described (Zhao, White et al. 2008). NARMS monitoring of food animals at slaughter was conducted by the USDA Bacterial Epidemiology and Antimicrobial Resistance Research Unit (BEAR) of the Agricultural Research Service (ARS) as previously described (Frye, Fedorka-Cray et al. 2008). Broth microdilution (Sensititre®, Trek Diagnostics Systems, Thermo Fisher Scientific Inc., Cleveland, OH) was used to determine the minimum inhibitory concentrations (MIC) for 15 antimicrobial agents. Resistance was defined by the Clinical and Laboratory Standards Institute (CLSI) interpretive standards, when available (CLSI 2013). For streptomycin, where no CLSI interpretive criteria for human isolates exist, the resistance breakpoint is 64 µg/ml (United States Food and Drug Administration (FDA) 2009). Testing was performed according to the manufacturer's instructions; the following quality control strains were used: *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 29213, *E. coli* ATCC 35218, and *Pseudomonas aeruginosa* ATCC 27853.

PCR amplification of *bla*_{CMY} genes

DNA templates for PCR was prepared by lysing the bacteria at 95°C and collecting the supernatant following centrifugation for 10 min at 20,000 g (Sorvall RC5B Plus, SS-34 rotor, Thermo Fischer Scientific Inc., Waltham, MA). PCR reactions contained 2x HotStar PCR Master Mix (Qiagen Inc., Valencia, CA), 0.4µM of each primer, 5µl template DNA and sterile PCR water to a final volume of 50µl. Thermal cycling was performed using the following conditions: 15 min at 95°C, followed by 30 cycles of 95°C for 30 s, 56°C for 30 s and 72°C for 90 s. To determine the presence of *bla*_{CMY} genes, primers *ampC1* (5'-ATGATGAAAAATCGTTATGC-3') and *ampC2* (5'-TTGCAGCTTTTCAAGAATGCGC-3') were used (Winokur, Vonstein et al. 2001).

Plasmid purification and characterization

Plasmids were purified using the QiaFilter Midi kit (Qiagen Inc.), following a modified manufacturer's protocol (Folster, Pecic et al. 2010). Electroporation of each plasmid into *E. coli* DH10B Electromax competent cells (Invitrogen, Carlsbad, CA) was performed as previously described (Folster, Pecic et al. 2010). Cells were plated on LB agar plates containing 100 mg/L of ampicillin or 4 mg/L ceftriaxone (Sigma-Aldrich, St. Louis, MO). All transformants were confirmed as *bla*_{CMY} positive by PCR analysis using primers *ampC1* and *ampC2*. DNA templates for PCR from transformants were prepared as described above. Plasmid PCR-based replicon typing (PBRT) was performed as previously described (Carattoli, Bertini et al. 2005) on the transformants. Plasmid multi-locus sequence typing was performed on IncI1 plasmids as previously described (Garcia-Fernandez, Chiaretto et al. 2008). Sequencing was performed using Big Dye version 3.1 (Applied Biosystems, Foster City, CA) and sequence reactions were cleaned with Centri-sep plates (Princeton Separations, Adelphia, NJ). The reactions were electrophoresed through POP-7 polymer (Applied Biosystems) on a 3730 DNA Analyzer (Applied Biosystems) equipped with a 48-capillary, 50 cm array. Sequence analysis was performed using Lasergene 8 software (DNASTAR Inc, Madison, WI). Sequences were submitted to the plasmid multi locus sequence type (pMLST) web page (<http://pubmlst.org/plasmid/>) and the sequence type was determined.

Pulsed-Field Gel Electrophoresis (PFGE)

Two enzyme (*Xba*I and *Bln*I) PFGE was performed according to the CDC PulseNet protocol (Ribot, Fair et al. 2006, Jackson, Fedorka-Cray et al. 2007). Isolates were grown overnight on Trypticase Soy Agar with 5% defibrinated sheep blood (TSA-SB) (Becton Dickinson Biosciences). Bacterial cell concentration was adjusted by diluting with sterile cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0) to a turbidity measurement of 0.48–0.52 (Dade Microscan Turbidity Meter). Agarose-embedded cells were lysed by proteinase K treatment and extensively washed. Agarose plugs containing genomic DNA were digested with 50U of *Xba*I and *Bln*I restriction enzymes (New England Biolabs, Ipswich, MA) and incubated at 37°C for 2 hours. The fragments were then separated by PFGE using a CHEF Mapper (Bio-Rad Laboratories) with the following conditions and reagents: 1% SeaKem Gold agarose in 0.5% TBE buffer, voltage at 6 V/cm, run time at 18 hours with switch times ranging from 2.16 to 63.8 seconds, temperature at 14°C. *Salmonella enterica* ser. Braenderup H9812 was used as a molecular reference marker. PFGE profiles generated were submitted to the PulseNet national database administered by CDC (NARMS-FDA and NARMS-CDC) or USDA VetNet (NARMS-USDA). Gel images were captured using the GelDoc XR system (Bio-Rad Laboratories) and Quantity One 1-D analysis software (Bio-Rad Laboratories). Pattern analysis and UPGMA dendrogram generation were performed using BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium) with the Dice coefficient and tolerance of 1.5%.

Results

Identification of *bla*_{CMY}-positive *Salmonella* ser. Typhimurium isolates

NARMS received and performed antimicrobial susceptibility testing on 581 isolates of *Salmonella* Typhimurium from food animals, retail meat, and humans in 2008. Of these, 70 (12.0%) displayed resistance to ceftriaxone and amoxicillin-clavulanic acid, suggesting the presence of a *bla*_{CMY} gene. Of the 70 isolates, 34 (48.6%) were from retail meat, specifically, chicken breasts. Twenty-three isolates (23/70 [32.9%]) were from food animal samples; 17/23 [73.9%] from chickens and 6/23 [26.1%] from cattle. Thirteen isolates were recovered from clinically-ill humans; 2/13 [15.4%] from male patients and 11/13 [84.6%] from females. The median age was 29.9 years with a range of less than 1 year to 77 years. PCR-analysis confirmed that all 70 resistant isolates carried a *bla*_{CMY} gene. Besides resistance to ceftriaxone and amoxicillin/clavulanic acid, all 70 isolates were resistant to the additional β -lactams tested (ampicillin, cefoxitin, and ceftiofur) (Figure 1). The most common additional resistance observed among the isolates was to sulfisoxazole (n=68), tetracycline (n=64), streptomycin (n=24), and chloramphenicol (n=14). Resistance to kanamycin (n=9) and gentamicin (n=3) was less common and resistance to amikacin, ciprofloxacin, nalidixic acid, and sulphamethoxazole/trimethoprim was not observed.

Characterization of the *bla*_{CMY} plasmids

To determine if the *bla*_{CMY} genes were located on a plasmid or the chromosome, plasmid DNA preparations were used to transform competent *E. coli* by electroporation. PCR-analysis showed successful transfer of *bla*_{CMY} genes to *E. coli* for 59/70 (84.3%) from the ESC resistant Typhimurium isolates, suggesting that 11 isolates likely carried *bla*_{CMY}

chromosomally. PCR-based replicon typing (PBRT) performed on the transformants revealed that all 59 *bla*_{CMY}-plasmid positive Typhimurium isolates carried the *bla*_{CMY} gene on one of two plasmid types; 42/59 (71.2%) had IncI1- and 17/59 (28.8%) had IncA/C-*bla*_{CMY} plasmids (Figure 1). Among the 51 chicken/chicken breast isolates, 38 had IncI1-*bla*_{CMY} plasmids, 3 had IncA/C-*bla*_{CMY} plasmids, and 10 isolates had no *bla*_{CMY} plasmids. Among the six cattle isolates, all had IncA/C-*bla*_{CMY} plasmids. Among the 13 isolates from humans, 4 isolates had IncI1-*bla*_{CMY} plasmids, 8 isolates had IncA/C-*bla*_{CMY} plasmids, and one had no *bla*_{CMY} plasmid. IncI1 plasmids were compared using plasmid multi-sequence typing (pMLST) (Garcia-Fernandez, Chiaretto et al. 2008). All 42 IncI1-*bla*_{CMY} plasmids were sequence type 12 or very closely related. Three isolates had plasmids with an identical point mutation in the *piLL* allele (G56 to A56) while 2 other isolates had plasmids two identical point mutations in the *sogS* allele (A175 to G175 and T177 to A177). All 5 five of these isolates were isolated from chicken breasts.

Determining similarity by PFGE of the *bla*_{CMY}-positive isolates

Two-enzyme PFGE was used to evaluate the genetic relatedness of the *bla*_{CMY}-positive Typhimurium isolates from different sources (Figure 1). Of the 70 isolates, 56 (80%) had unique 2-enzyme patterns suggesting very little clonal spread. There were 9 groups of isolates with indistinguishable patterns and the largest group contained 4 isolates. The isolates grouped into 3 main clusters (labeled A, B, and C) and one outlier (bottom of dendrogram). The largest group, cluster C, contained closely related (> 75% with 2-enzymes) isolates from poultry sources (chicken breast [n=31] and chickens [n=16]) and humans [n=3]. None of these isolates displayed resistance to chloramphenicol. Isolates in cluster C primarily contained IncI1-*bla*_{CMY} plasmids (n=39/50; 78%) or no *bla*_{CMY} plasmids (8/50; 16%). Only three isolates contained IncA/C-*bla*_{CMY} plasmids. Cluster A was the next largest cluster with 15 isolates. Although not as related (< 75%) as cluster C, cluster A contained almost all of the isolates with chloramphenicol resistance (12/14; 85.7%) and most of the isolates with streptomycin resistance (14/24; 58.3%), meeting the definition of MDR-AmpC (Harbottle, White et al. 2006). Isolates in cluster A primarily contained isolates of either human (n=9) or cattle (n=5) sources and only a single poultry isolate. Cluster A also consisted primarily of isolates with IncA/C-*bla*_{CMY} plasmids (12/15; 80%) with only two IncI1-*bla*_{CMY} containing isolates and a single isolate with no *bla*_{CMY} plasmid. Cluster B contains only 4 isolates and is mixed with respect to antimicrobial resistance patterns (two isolates displayed chloramphenicol resistance), isolate source (two isolates from chicken breast, one cattle, and one human source) and *bla*_{CMY} plasmid type (two IncA/C-*bla*_{CMY}, one IncI1-*bla*_{CMY}, and one without a *bla*_{CMY} plasmid).

Discussion

Laboratory-based disease surveillance and foodborne outbreak detection and investigation requires high quality epidemiological data and detailed agent information. With the evolution of new strain typing techniques, investigators have sought to combine various tools to provide more specific agent information in an effort to improve the surveillance and investigative processes. We examined the value of combining phenotypic data on

antimicrobial resistance and serotype with genetic data represented by PFGE patterns and differences in plasmid content.

IncI1 plasmids are a narrow-host-range plasmid type and are limited to enteric bacteria (Johnson, Shepard et al. 2011). They are commonly associated with *Salmonella* and *E. coli* from avian and porcine sources, and are more frequent among pathogenic than commensal *E. coli* strains found among avian and human sources in the U.S. (Johnson, Wannemuehler et al. 2007). IncI1 plasmids commonly carry genes conferring ESC resistance, including *bla*_{CMY} and *bla*_{CTX-M}. Previous studies have identified IncI1 plasmids as the primary plasmid type carrying *bla*_{CMY} among humans with *Salmonella* serotypes commonly associated with poultry and IncI1 is the most prominent *bla*_{CMY} plasmid type among serotype Heidelberg isolates from humans and poultry sources (Folster, Pecic et al. 2010, Folster, Pecic et al. 2011).

IncA/C plasmids have a broad host range and have been isolated from diverse groups of Proteobacteria found in the environment, animals, and humans (Lang, Danzeisen et al. 2012). IncA/C plasmids are commonly large, multidrug resistant, and have been identified among isolates from food animals including beef, chicken, turkey and pork, suggesting that they may be responsible for the spread of MDR from food animals to humans (Mulvey, Susky et al. 2009). IncA/C plasmids are one of the most frequent plasmid types carrying *bla*_{CMY} in the United States (Giles, Benson et al. 2004). IncA/C plasmids are the most common plasmid type carrying *bla*_{CMY} among humans with *Salmonella* serotypes usually associated with cattle and beef sources, including Newport and Dublin (Folster, Pecic et al. 2010). A study of *bla*_{CMY} plasmids from *E. coli* and *Salmonella* in Canada found that bacteria from cattle and beef all had IncA/C plasmids (Martin, Weir et al. 2012).

In this study, we identified and characterized 70 ceftriaxone and amoxicillin/clavulanic acid resistant *Salmonella* ser. Typhimurium isolates. All of the isolates contained a *bla*_{CMY} gene and over 80% were plasmid encoded. We identified two *bla*_{CMY} plasmid types, IncI1 and IncA/C, with IncI1 comprising greater than 70% of *bla*_{CMY} plasmids identified. When we compared plasmid type to the source of each non-clinical isolate we found that nearly all of the isolates with *bla*_{CMY} plasmids from chicken or chicken products had IncI1-*bla*_{CMY} plasmids (92.5%), while all of the cattle isolates had IncA/C-*bla*_{CMY} plasmids. This shows a correlation between the animal source of Typhimurium isolates and the replicon type of *bla*_{CMY} plasmid they carry. However, a larger study set of isolates over several years is needed to confirm this observation. Clinical isolates from humans with *bla*_{CMY} plasmids had both IncA/C-*bla*_{CMY} (n=8) and IncI1-*bla*_{CMY} (n=4) plasmids; however, no information was available regarding the source of infection of these routine surveillance isolates.

When we compared plasmid type, source, and antimicrobial resistance patterns we found that all of the IncA/C-*bla*_{CMY} isolates from cattle and humans were resistant to chloramphenicol. Chloramphenicol resistance is commonly conferred by IncA/C plasmids (Lindsey, Frye et al. 2011). However, the three IncA/C-*bla*_{CMY} isolates from chicken breasts were all chloramphenicol susceptible and none of the IncI1-*bla*_{CMY} positive isolates from chicken or chicken breasts were chloramphenicol resistant. This suggests that

chloramphenicol resistance, when observed along with the *bla*_{CMY} mediated resistance phenotype, may point to a possible cattle/beef source.

PFGE analysis showed a significant amount of genetic variation among all 70 isolates but the isolates grouped into 3 main clusters (A, B, and C). When we compared this to isolate source, *bla*_{CMY} plasmid type, and chloramphenicol resistance, we again found a strong correlation. Cluster C, the largest cluster with 50 isolates, contained 92.2% of the poultry isolates and no cattle isolates, 91.6% of the IncII-*bla*_{CMY} plasmids, and no chloramphenicol resistant isolates. In contrast, cluster A, containing 15 isolates, had five out of six isolates from cattle, 70.6% (12/17) of the IncA/C-*bla*_{CMY} plasmids, and 85.7% (12/14) of the chloramphenicol resistant isolates. If we further divide cluster A, the bottom group (containing seven isolates) contains isolates with an identical resistance profile and plasmid type (IncA/C) but isolates from both cattle (n=4) and humans (n=3), suggesting that the human isolates were likely acquired from a beef source.

Interestingly, even though *Salmonella* is rarely isolated from ground beef and none of the isolates in this study were isolated from ground beef, the majority of isolates from humans had IncA/C-*bla*_{CMY} plasmids, which we interpret to indicate that humans were more likely to acquire *bla*_{CMY} isolates with plasmids that resemble those from cattle sources than poultry sources. This suggests that human infections with *bla*_{CMY} positive Typhimurium isolates may be from another beef source which is not being sampled or that current sampling and/or isolation methods are not detecting *Salmonella* in ground beef. The latter may be due to processing events specific for ground beef which could result in a temporary decrease in *Salmonella* numbers at the time of sampling (Harris, Brashears et al. 2012). It is also possible that humans are acquiring *bla*_{CMY} positive *Salmonella* ser. Typhimurium from unsampled non-meat sources, including vegetables.

Conclusions

Although the numbers of isolates in the study were small, especially from cattle, we did find associations between *bla*_{CMY} plasmid type and food animal source (IncA/C and cattle, IncII and poultry). This work may be useful in generating hypotheses about the sources for foodborne outbreaks and sporadic illness caused by *Salmonella*. However, resistance plasmid type needs to be examined along with careful consideration of all of the available information. This is particularly important for some serotypes like Typhimurium which has great genetic diversity and wide range of hosts. Further work is necessary to determine if these correlations occur with other *Salmonella* serotypes, other enteric bacteria, and additional antimicrobial resistance plasmids. Analysis of a larger set of isolates over a longer period of time is needed to determine how best to combine plasmid, PFGE, serotype, and susceptibility data to effectively guide investigations of foodborne disease.

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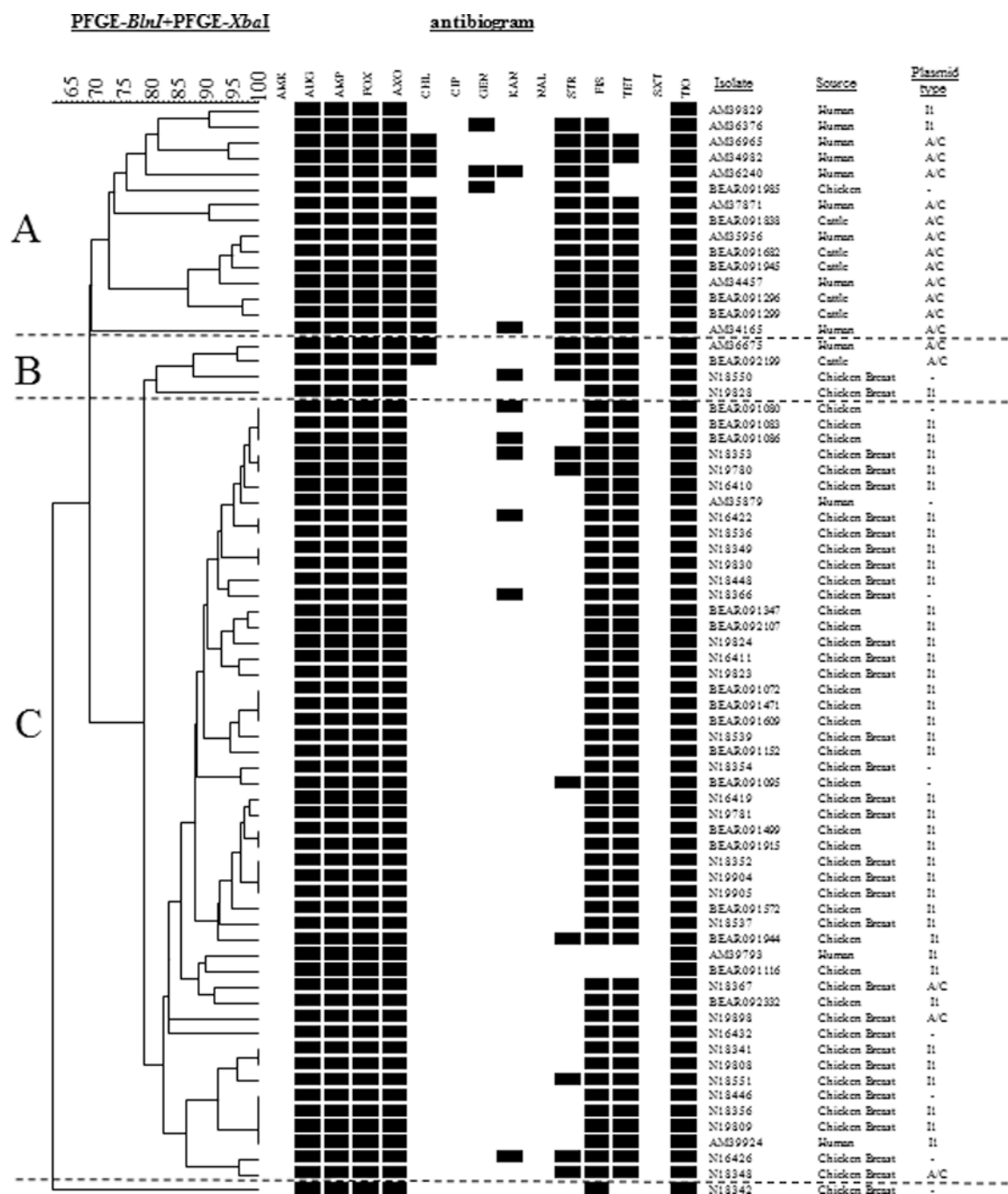


Figure 1.

PFGE patterns of *bla*_{CMY}-positive *Salmonella enterica* ser. Typhimurium isolated from food animals, retail meat, and humans in the United States in 2008. Dendrogram of percent genetic similarity by PFGE was generated using BioNumerics based on *XbaI* and *BlnI* restriction digestion. Pattern analysis and UPGMA dendrogram generation were performed using BioNumerics software (Applied Maths, Saint-Martens-Latem, Belgium) with the Dice coefficient and tolerance of 1.5%. Percent similarity is located above dendrogram. Antibiogram displays the antimicrobial resistance profile of the isolates; a black box

indicates resistance to that antimicrobial. AMK, amikacin; AUG, amoxicillin/clavulanic acid; AMP, ampicillin; FOX, ceftiofur; AXO, ceftriaxone; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; FIS, sulfisoxazole; TET, tetracycline; SXT, trimethoprim-sulfamethoxazole; TIO, ceftiofur. Isolate number, source, and plasmid incompatibility type are listed to the right of the antibiogram. The “-“symbol represents that no *bla*_{CMY} plasmid was found. The isolates are grouped into three clusters, labeled A, B, and C.